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COVALENT BINDING OF 4-DIMETHYLAMINOPHENYLAZO-^{3}H-BENZENE (BUTTER YELLOW) METABOLITES WITH LIVER RIBOSOMAL RNA: THE DISSOCIATION OF THE BINDING MECHANISM FROM THE OROTIC ACID INCORPORATING SYSTEM

by

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1. No overall correlation between the binding of ^{3}H-labelled metabolites of 4-dimethylaminophenylazobenzene (DAB) to the r-RNA of liver, spleen and kidney and the incorporation of orotic acid-^{14}C into the same fractions after the same time was observed.

2. While actinomycin D inhibited the incorporation of orotic acid-^{14}C into liver r-RNA it did not cause a similar depression in the binding of ^{3}H-labelled metabolites of DAB to r-RNA.

3. Actinomycin D did not affect the overall metabolism of DAB as judged by the binding of ^{3}H-labelled metabolites to liver cytoplasmic and nuclear protein fractions.

4. These results suggest that the association of radioactivity with liver r-RNA following injection of DAB-^{3}H is mediated by reactions independent of the orotic acid incorporation mechanism. They also confirm the earlier findings that reaction with liver r-RNA is particularly favoured compared to that with r-RNA from other sites.

A previous publication (Roberts and Warwick, 1966*a*) demonstrated that radioactive metabolites of DAB-^{3}H were covalently bound to liver r-RNA following an intraperitoneal (i.p.) injection. Furthermore, an association between the susceptibility of a particular tissue to undergo malignant transformation and the level of the radioactivity associated with its r-RNA appeared to exist; also the level of the binding to r-RNA of azo-dye-induced transplanted hepatomas relative to that of the livers in the same animals was found to be diminished. One possible explanation for the differences in the levels of reaction of butter yellow metabolites with various r-RNAs is that a metabolite of DAB could conceivably be incorporated into r-RNA by reacting with a RNA precursor rather than be bound by a direct reaction with preformed r-RNA. In this case the degree of labelling of r-RNA would be directly related to the rate and extent of r-RNA synthesis in the various tissues. Moreover, there could be differences in the extent to which different tissues are capable of utilizing such precursors, since Wilkinson and Kirby (1966) have demonstrated that hepatoma tissue incorporated RNA precursors less efficiently than liver.

We therefore investigated this possibility by comparing the level of incorporation of orotic

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acid-^{14}C into the r-RNA of liver, spleen and kidney with the level of binding of ^{3}H-labelled metabolites of DAB to these ribonucleic acids. The effect of actinomycin D administered in doses sufficient to depress the rate of ribosomal RNA synthesis on the binding of metabolites of DAB was also determined.

MATERIAL AND METHODS

Orotic acid-^{14}C was supplied by the Radiochemical Centre (Amersham, Bucks, England), at 30 mc/mmole and was administered at a dose of 50 μc/kg in water (1 ml) by i.p. injection.

DAB-^{3}H (dimethylaminophenylazobenzene-^{3}H; benzene moiety generally labelled) was prepared as described previously (Roberts and Warwick, 1966*a*), diluted with unlabelled butter yellow to a specific activity of 1 mc/mg, and administered at a dose of 150 mg/kg in arachis oil (2 ml).

Actinomycin D was injected i.p. in 8% aqueous propylene glycol solution (0.5 ml) at doses ranging from 0.25 to 1 mg/kg.

Male Wistar rats weighing 150-250 g were maintained on a diet of 10% protein for at least a week prior to injection. DNA, r-RNA and cytoplasmic and nuclear protein fractions from liver, spleen and kidney tissues were isolated by a recent modification (Kirby, 1965) of the process of Kidson *et al.* (1963). DNA and r-RNA were purified by the methods of Roberts and Warwick (1966*a*) and the radioactive content of proteins and nucleic acid fractions was determined after pretreatment as described using a three-channel liquid scintillation counter with automatic external standardization (Model 3003, Packard Instrument Corp., Downers Grove, Ill., USA).

In all experiments the binding of ^{3}H-labelled metabolites to cellular constituents was determined 17 hr after injection of DAB-^{3}H, i.e. the time taken for maximum binding to occur in the liver, as previously determined (Roberts and Warwick, 1966*a*). The incorporation of orotic acid-^{14}C into r-RNA was also determined after the same time interval. Individual rats were used for the isolation of the various cellular fractions from liver, spleen, and kidney and the results are expressed as the average of ratios obtained from at least two animals.

RESULTS

Incorporation of orotic acid-^{14}C into r-RNA of rat liver, spleen and kidney was determined after 17 hr. As illustrated in Figure 1, the specific radioactivity of liver r-RNA was approximately twice that of kidney r-RNA and 20 times that of spleen r-RNA. These values are compared in the same Figure with the amount of ^{3}H bound to similar RNA fractions at the same time after i.p. injection of DAB-^{3}H. It was evident that the specific radioactivity of liver r-RNA was again 20 times that of spleen r-RNA. The similarity between these ratios could therefore be due to much more efficient uptake of both orotic acid and DAB into liver compared with spleen. Alternatively the rate of synthesis of RNA could be 20 times greater in liver than in spleen. In order to account for the greater

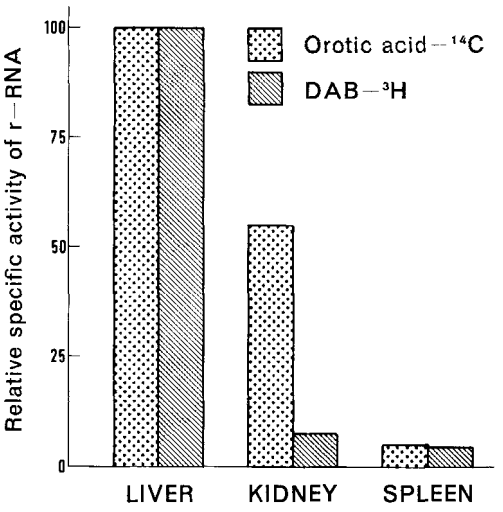

FIGURE 1

INCORPORATION OF OROTIC ACID-^{14}C AND BINDING OF DAB-^{3}H TO r-RNA.

Relative amounts of ^{14}C incorporated into rat liver, kidney and spleen r-RNA 17 hr after i.p. injection of orotic acid-^{14}C [50 μc/kg: specific activity 30 mc/ mmole] and the relative amounts of reaction of ^{3}H-labelled metabolites of DAB with similar fractions, the same time after i.p. injection of DAB-^{3}H [150 mg/kg: specific activity 1 mc/mg]. The results are expressed as the average of ratios determined on two individual rats. The specific radioactivity of ^{14}C-labelled rat liver r-RNA was taken as 100 which was equivalent to 21 μc/g, and the specific radioactivity of ^{3}H-labelled rat liver r-RNA was also taken as 100 which was equivalent to 0.12 μmole/g.

binding of DAB to liver in terms of this difference alone it would then be necessary to assume that DAB was either bound to or incorporated into an RNA precursor. However for kidney, the quantity of orotic acid incorporated into r-RNA was much greater than that of metabolites of DAB bound to it. This suggests that the differences in specific radioactivities of ^{3}H-labelled r-RNAs of the various organs after injection of DAB-^{3}H are not solely due to differences in the availability of possible RNA precursors.

a dose of 0.25 mg/kg the amount of orotic acid-^{14}C incorporated into liver r-RNA was reduced to 43% of the control value during 17 hr while at a dose of 0.5 mg this was further reduced to 12% of the control. This latter dose led to the death of animals between 24 and 36 hr after the injection, while a dose of 1 mg/kg resulted in death within 16 hr. The incorporation of orotic acid-^{14}C into kidney r-RNA was similarly reduced by an amount comparable to that found in the liver after injection of actinomycin D

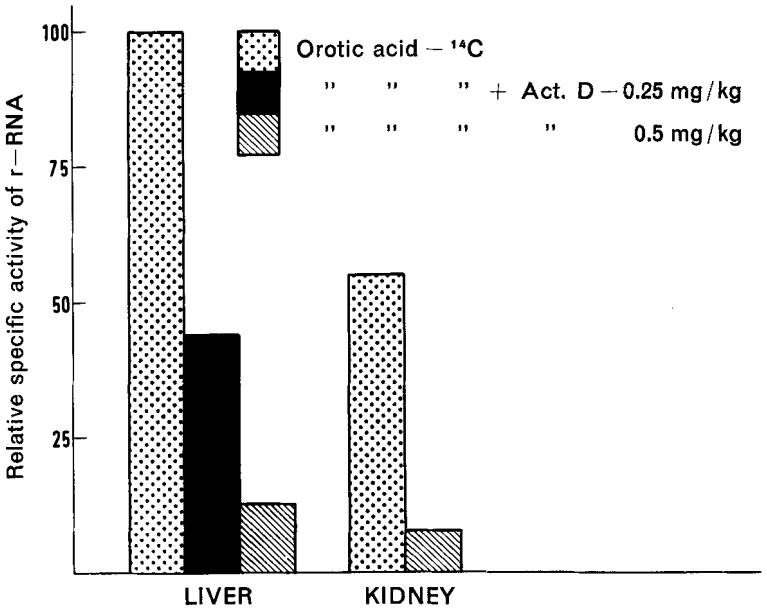

FIGURE 2

EFFECT OF ACTINOMYCIN D ON INCORPORATION OF OROTIC ACID-^{14}C INTO r-RNA

The effect of actinomycin D (0.25 and 0.5 mg/kg) on the incorporation of radioactivity into r-RNA of rat liver, spleen and kidney 17 hr after i.p. injection of orotic acid-^{14}C [50 μc/kg: specific activity 30 mc/mmole]. In both sets of experiments the actinomycin was administered 5 min before the orotic acid.

In order to test further whether the differences were due to variations in the rates of RNA synthesis in the various organs, these rates were modified by the simultaneous administration of actinomycin D, and the amounts of ^{3}H-metabolites bound to the different RNAs were compared. The effect of administration of actinomycin D on the incorporation of orotic acid-^{14}C into r-RNA of liver and kidney was first investigated. The results are illustrated in Figure 2. At

at 0.5 mg/kg. The incorporation of orotic acid-^{14}C into spleen r-RNA was at too low a level to permit the effect of added actinomycin D to be studied.

Initial experiments designed to test the effect of actinomycin D on the binding of ^{3}H-labelled metabolites of butter yellow to liver r-RNA were carried out using 0.5 mg/kg of actinomycin D administered 10 min or 60 min prior to injection of the DAB-^{3}H. However, the precise degree of

inhibition of RNA synthesis would not necessarily be the same under these conditions as that found in the absence of butter yellow (see Fig. 2). The use of orotic acid-^{14}C in conjunction with DAB-^{3}H indicated that the degree of inhibition was in fact only 50% of the control value. By increasing the dose of actinomycin D to 0.625 mg/kg and administering it 2 hr prior to the injection of DAB the degree of inhibition of RNA synthesis in the liver was reduced to 16% that of the control. At this concentration of actinomycin D the incorporation of orotic acid-^{14}C into kidney r-RNA was similarly depressed to a value of 3% of the control. In these experiments suitable controls were considered to be the

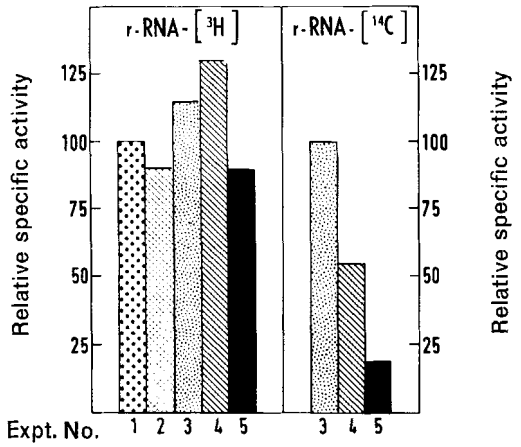

FIGURE 3

EFFECT OF ACTINOMYCIN D ON BINDING OF DAB-^{3}H TO r-RNA

The effect of actinomycin D on the incorporation of ^{14}C or ^{3}H into liver r-RNA after injection of either orotic acid-^{14}C or DAB-^{3}H. The experimental conditions were as follows:

1. DAB-^{3}H (150 mg/kg) alone.
2. Actinomycin D (0.625 mg/kg) given 120 min prior to DAB-^{3}H (150 mg/kg).
3. Orotic acid-^{14}C (50 μc/kg) 60 min prior to DAB-^{3}H (150 mg/kg).
4. Actinomycin D (0.5 mg/kg) and orotic acid-^{14}C (50 μc/kg) given 10 min and 2 min respectively prior to DAB-^{3}H (150 mg/kg).
5. Actinomycin D (0.625 mg/kg) and orotic acid-^{14}C given 120 min and 60 min respectively prior to DAB-^{3}H (150 mg/kg).

The specific radioactivity of ^{14}C-labelled r-RNA in Experiment 3 was the same as under the conditions in Figure 1 in the absence of DAB-^{3}H and the results are expressed as this value equal to 100. The specific radioactivity of liver r-RNA after injection of DAB-^{3}H alone was expressed as 100.

binding of DAB-^{3}H metabolites to cellular constituents of rats which had received DAB-^{3}H alone, DAB-^{3}H after actinomycin D only, DAB-^{3}H after orotic acid-^{14}C only and finally of rats which had received DAB-^{3}H after injection of actinomycin D and orotic acid-^{14}C. The results, illustrated in Figure 3, indicate that even when RNA synthesis in the liver was depressed to 16% of the control value there was no significant difference in the level of binding of DAB-^{3}H metabolites bound to liver r-RNA as compared with the amounts bound to the r-RNAs of the livers of control animals.

The value found for the binding of butter yellow metabolites to kidney r-RNA was extremely low and approximately equal to that found for the binding of metabolites to the r-RNA isolated from spleen (Fig. 1). In view of these low values it was not possible to be certain whether or not actinomycin D depressed the binding of DAB metabolites to spleen or kidney r-RNA.

The binding of ^{3}H-labelled metabolites of DAB to protein fractions of liver, spleen and kidney was also determined in the above experiments. This was done to establish that prior injection of actinomycin D did not diminish the degree of metabolism of DAB to reactive intermediates, since it is possible that actinomycin D could cause a depression in the synthesis of messenger RNAs associated with the production of metabolizing enzymes induced by the administration of butter yellow; cf., for example, studies on the effect of actinomycin D on the induction of enzymes by hydrocarbons (Loeb and Gelboin, 1964; Gelboin and Blackburn, 1964). The results, as illustrated in Figure 4, indicated however that the binding of ^{3}H-labelled metabolites of DAB to cytoplasmic and nuclear proteins of the liver was unaltered by prior administration of actinomycin D. Figure 4 also indicates that while the amount of reaction with spleen and kidney cytoplasmic protein fractions was greater than that with liver cytoplasmic protein, after prior injection of actinomycin D there was evidence that the amount bound to these fractions was diminished. It is more probable that the time course of labelling of these proteins could have been modified by the action of actinomycin D than that any absolute reduction had occurred in the amount of label bound to them.

A combination of the data in Figures 1 and 4 indicates that the extent of binding of ^{3}H-labelled metabolites of DAB to kidney cytoplasmic protein is nearly 30 times higher than to kidney RNA, compared with the corresponding ratio for liver of around 5:1. Such a high value for kidney means that it is difficult to exclude the possibility that such radioactivity present on RNA could be due to its contamination with protein. Similarly the binding of ^{3}H-labelled metabolites to kidney DNA, which was of the same order as that found to kidney r-RNA, could also be due to the presence of ~3% residual protein of the same specific activity as that of kidney cytoplasmic protein. This possibility appears, however, to be excluded by the results of parallel studies, to be reported in full elsewhere (Warwick and Roberts, 1966*b*, *c*) on the reaction of DAB metabolites with DNA. It was found that radioactivity remained bound to DNA for longer than would be expected had it been due to a protein contaminant, assuming that the radioactivity bound to the latter would turn over at the same rate as the radioactivity present on the cytoplasmic protein fraction. It would seem therefore that any radioactivity associated with the DNA fraction as isolated is the result of reaction of a metabolite of DAB with DNA itself.

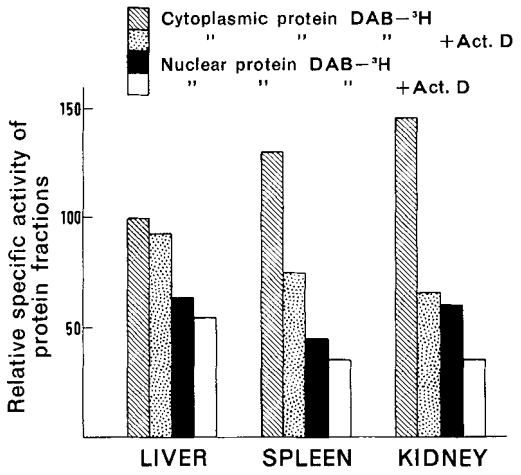

FIGURE 4

EFFECT OF ACTINOMYCIN D ON BINDING OF DAB-^{3}H TO PROTEINS

The effect of actinomycin D on the binding of ^{3}H-labelled metabolites of DAB to liver, spleen and kidney cytoplasmic and nuclear protein fractions. The experimental conditions were the same as those for Experiments 1 and 2, Figure 3. The specific radioactivity of liver cytoplasmic protein after injection of DAB-^{3}H (150 mg/kg) alone was expressed as 100, and was equivalent to 0.55 μmole/g.

DISCUSSION

The differences in the degree of incorporation of orotic acid-^{14}C into the various r-RNAs examined are a measure either of the extent to which orotic acid is utilized in the various organs and/or a measure of differences in the rates of RNA synthesis taking place. Such factors may also account for the observed differences in the binding of ^{3}H-labelled metabolites of DAB to the different RNAs if DAB were bound to an RNA precursor or converted into such a precursor. The lack of correlation between the amount of orotic acid-^{14}C incorporated into r-RNA of liver, kidney and spleen and the binding of ^{3}H-labelled metabolites of DAB to these same fractions after the same time would suggest that this explanation is not tenable. Furthermore, while actinomycin D can inhibit the incorporation of orotic acid-^{14}C into liver r-RNA, no corresponding inhibition in the binding of ^{3}H-labelled metabolites of DAB results, as would be the case if metabolites of DAB were biosynthetically incorporated into r-RNA in this manner. The radioactivity associated with r-RNA after DAB administration must therefore result from direct reaction of a metabolite or metabolites with r-RNA and is not associated with the orotic acid incorporation mechanism. The fact that the binding of ^{3}H-labelled metabolites of DAB to the DNA fractions of liver, spleen and kidney is similar and comparable to the binding to r-RNA of spleen and kidney suggests that the much higher level of binding to the r-RNA of liver is the result of some special mechanism. In liver the enzymes which activate DAB may be located near ribosomes whereas in spleen or kidney this might not be the case.

On the basis of current theories of the mechanism of chemical carcinogenesis the evidence obtained so far in these studies is that while a certain critical level of reaction with DNA is probably essential for the initiation of the carcinogenic process, chemical alteration of r-RNA and proteins or other cytoplasmic constituents may also be required for the subsequent promotion which is a prerequisite of tumour development (cf. Potter, 1964).

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LIAISON COVALENTE DU 4-DIMÉTHYLAMINOPHÉNYLAZO-^{3}H-BENZÈNE (MÉTABOLITES DU JAUNE DE BEURRE) AVEC L'ARN RIBOSOMIQUE DU FOIE: DISSOCIATION DU MÉCANISME DE LIAISON ET DU SYSTÈME INCORPORANT L'ACIDE OROTIQUE

On n'a observé aucune corrélation générale entre, d'une part, la liaison covalente de métabolites du 4-diméthylaminophénylazobenzène (DAB) marqués au tritium avec l'ARN ribosomique du foie, de la rate et du rein et, d'autre part, l'incorporation d'acide orotique-^{14}C aux mêmes fractions après le même temps.

Alors que l'actinomycine D inhibait l'incorporation d'acide orotique-^{14}C à l'ARN ribosomique du foie, elle n'abaissait pas de façon analogue la liaison des métabolites du DAB marqués au ^{3}H avec l'ARN ribosomique.

L'actinomycine D n'influençait pas le métabolisme global du DAB, à en juger par la liaison des métabolites marqués au ^{3}H avec les fractions protéiniques du cytoplasme et du noyau des cellules hépatiques.

Ces résultats font penser que l'association de la radioactivité avec l'ARN ribosomique du foie après injection de DAB-^{3}H s'effectue par des réactions indépendantes du mécanisme d'incorporation de l'acide orotique. Ils confirment, d'autre part, les observations antérieures montrant que la réaction avec l'ARN ribosomique du foie est particulièrement favorisée par rapport à la réaction avec celui d'autres organes.

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